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Development and Validation of a Rotor-Gene Real-Time PCR Assay for Detection, Identification, and Quantification of *Chlamydia trachomatis* in a Single Reaction

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A multitarget real-time PCR (MRT-PCR) for detection of *Chlamydia trachomatis* DNA was developed and validated. There were three targets for amplification in a single reaction: the cryptic plasmid (CP), the major outer membrane protein (MOMP) gene, and an internal control. The assay had the following characteristics: (i) detection and confirmation of the presence of *C. trachomatis* DNA in a single reaction, (ii) detection of all genovars of *C. trachomatis* without any cross-reactivity with pathogenic bacteria or commensal organisms of the oropharynx and genital tract, (iii) a 95% probability of detection with three copies of MOMP and one copy of CP per reaction mixture, (iv) identification of the inhibition of amplification, (v) a quantitative dynamic range of 25 to 250,000 genome copies per reaction mixture, (vi) high intra- and interassay reproducibilities, and (vii) correct identification of all samples in the validation panel. There were 146 COBAS Amplicor PCR (Amplicor PCR)-positive samples and 122 Amplicor PCR-negative samples in the panel. MRT-PCR detected CP DNA alone in 6 (4%) Amplicor PCR-positive samples and both CP and MOMP DNAs in 140 (96%) of 146 Amplicor PCR-positive samples. The quantity of MOMP DNA in 95 (68%) of 140 samples was within the dynamic range of the assay. The median *C. trachomatis* load in these samples was 321 genome copies per reaction mixture (range, 26 to 40,137 genome copies per reaction mixture). Due to the inclusion of two different *C. trachomatis*-specific targets, the assay confirmed 259 (97%) of 268 results in a single reaction. This assay could be used in the qualitative format for the routine detection of *C. trachomatis* and in the quantitative format for study of the pathogenesis of *C. trachomatis*-associated diseases. The assay demonstrated the potential to eliminate the need for confirmatory testing in almost all samples, thus reducing the turnaround time and the workload.

Chlamydia trachomatis is a bacterial infection of global public health significance (10). It is an intracellular pathogen with 15 serotypes. It causes trachoma (serotypes A, B, B_1, and C), lymphogranuloma venereum (serotypes L_1, L_2, and L_3), and oculogenital infections (serotypes D to K). There are many highly conserved nucleotide sequences in different chromosomal genes and in the cryptic plasmid (CP) of *C. trachomatis*. These sequences are used as targets for nucleic acid amplification tests (NAATs). NAATs have surpassed cell culture and antigen detection for the diagnosis of *C. trachomatis* infections due to their enhanced sensitivities.

A large number of in-house NAATs for the detection of *C. trachomatis* have been reported (16). The majority of these are gel based. A few non-gel-based NAATs have also been described, i.e., a Qβ replicase-amplified assay (2) and PCR-enzyme immunoassays (4, 11, 21). All previously reported in-house NAATs are manual and use open systems with separate steps for amplification and amplicon analysis. Due to the risk of contamination and a lack of automation, these assays are inappropriate for diagnostic laboratories with high workloads. Real-time PCR is performed in a closed system, amplification and detection of target are done in a single step, and there is the potential for automation. Real-time PCR has been used for pharmacodynamic studies for *C. trachomatis* (19, 23). Two real-time PCR assays for the detection of *C. trachomatis* have also been described (8, 13). Both these assays used the SYBR green technology in the LightCycler system (Roche Molecular Systems, Inc., Pleasanton, CA) and were used for the qualitative detection of *C. trachomatis*. Roche Molecular Systems, GEN-PROBE Inc. (San Diego, CA), BD Diagnostics (Sparks, MD), and artus GmbH (Hamburg, Germany) are the four major commercial companies which supply NAATs for the detection of *C. trachomatis* in Europe. At present, artus GmbH is the only commercial company that provides an assay for the quantification of *C. trachomatis* in clinical samples.

A single gene of *C. trachomatis* per amplification reaction was targeted in all of the in-house and commercial assays mentioned above. The targets of amplification were the CP, the major outer membrane protein (MOMP) gene, the 23S rRNA gene, and the cysteine-rich outer membrane protein gene (16). In the present study, we report on a multitarget real-time PCR (MTR-PCR) assay for the detection, identification, and quantification of *C. trachomatis* in a single reaction. Three targets of amplification were used in this assay: the

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TABLE 1. Chlamydia strains

Strains	ATCC code
LGV I strain 440	VR-901B
LGV II strain 434	VR-902B
LGV III strain 404	VR-903
A strain HAR-13	VR-571B
B strain HAR-36	VR-573
C strain TW-3	VR-1477
D strainUW-36/Cx	VR-885
E strain BOUR	VR-348B
F strain IC-Cal-3	VR-346
G strain UW-57/Cx	VR-878
H strain UW-43/Cx	VR-879
I strain UW-12/Ur	VR-880
J strain UW-36/Cx	VR-886
K strain UW-31/Cx	VR-887
C. suis strain S45	VR-1474
C. pneumoniae	CDC-CWL-011

cryptic plasmid for the qualitative detection, the MOMP gene for the quantification of *C. trachomatis* DNA, and an internal control (IC) for the detection of inhibitors of amplification. None of the previously reported in-house or commercial assays have achieved these characteristics in a single reaction.

MATERIALS AND METHODS

***Chlamydia* strains.** Strains of *C. trachomatis* from different serovars and *C. suis* were grown in McCoy cells, as described previously (25). The growth of the organism was confirmed by direct immunofluorescence with a genus-specific monoclonal antibody, IMAGEN Chlamydia, according to the manufacturer's protocol (DakoCytomation Ltd., Ely, United Kingdom). McCoy cells and all strains of chlamydia were obtained from the American Type Culture Collection (ATCC), LGC Promochem, Middlesex, United Kingdom. However, *C. pneumoniae* was not grown in the cell culture; its DNA was obtained from Advanced Biotechnologies, Incorporated (Columbia, MD). The identities of the *Chlamydia* strains used in this study are shown in Table 1.

Bacterial strains. Pathogenic bacteria and commensal organisms of the oropharynx and the genital tract were grown as described previously (1). The suitability of bacterial DNA for amplification was checked by 16S rRNA gene-based PCR (15). The identities of the bacterial strains used in this study are shown in Table 2.

Validation panel. The validation panel included 146 Amplicor PCR-positive and 122 Amplicor PCR-negative samples. These samples were obtained from 56 males (urethral swabs; median age, 22 years; age range, 17 to 64 years) and 212 females (both urethral and endocervical swabs in a single tube; median age, 23 years; age range, 15 to 59 years) as described previously (12). The samples were coded, and the results of the Amplicor PCR were masked before the validation experiments. The swab in the transport medium was agitated for 20 s at a setting of 4.5 in a multitube vortexer (model 2601; Scientific Manufacturing Industries, Emeryville, CA) before extraction of DNA. The DNA from 200 μl of cell suspension (a bacterial culture or a clinical sample) was extracted by using a MagNA Pure LC total nucleic acid isolation kit and MagNA Pure LC Robot, according to the manufacturer's protocol (Roche Molecular Systems). DNA was concentrated during extraction and eluted in 100 μl of elution buffer. Quality control for DNA extraction was performed by inclusion of *C. trachomatis* culture genotype E (positive control) and nuclease-free water (negative control) in each run.

PCR. Real-time PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, Australia). Rotor-Gene has four light-emitting diodes that allow the detection of up to four targets in a single amplification reaction. This characteristic of the system was used to develop the MTR-PCR for the detection of *C. trachomatis*. A pair of primers and a labeled probe in the TaqMan format were used for each component of the assay, i.e., CP, MOMP, and IC. Except for the probe for the MOMP gene, the IC DNA and all primers and probes were synthesized by Metabion International AGi, Germany. The probe for the MOMP gene was obtained from Applied Biosystems, Cheshire, United Kingdom. The sequences of the primers, probes, and the internal control are shown in Table 3. PCR was performed in a 25-μl reaction mixture containing 10 μl of DNA from a clinical sample or a bacterial isolate, 5 mM $MgCl_2$, 12.5 μl of

TABLE 2. Specificity of real-time PCR for cryptic plasmid and MOMP components of the assay

Bacterium	Code	PCR result			
		16S rRNA PCR	Real-time PCR for *Chlamydia*		
			IC	Plasmid	MOMP
Streptococcus mutans	ATCC 25175	+	+	−	−
Streptococcus canis	ATCC 3497	+	+	−	−
Streptococcus sanguis	ATCC 10556	+	+	−	−
Staphylococcus aureus	ATCC 25923	+	+	−	−
Staphylococcus epidermidis	ATCC 12228	+	+	−	−
Neisseria meningitidis	ATCC 13077	+	+	−	−
Neisseria gonorrhoeae	NCTC 8375	+	+	−	−
Escherichia coli	NCTC 10418	+	+	−	−
Cornyebacterium ulcerans	NCTC 7907	+	+	−	−
Pasteurella haemolytica	NCTC 9380	+	+	−	−
Fusobacterium necrophorum	NCTC 10576	+	+	−	−
Yersinia enterocolitica	ATCC 23715	+	+	−	−
Cornyebacterium diptheriae	ATCC 11913	+	+	−	−
Arcanobacterium haemolyticum	NCTC 8452	+	+	−	−
Prevotella buccae	ATCC 33574	+	+	−	−
Veillonella parvula	ATCC 10790	+	+	−	−
Fusobacterium ulcerans	NCTC 12111	+	+	−	−
Prevotella melaninogenica	ATCC 25845	+	+	−	−
Actinobacillus actinomycetemcomitans	ATCC 29522	+	+	−	−
Actinomyces israelii	ATCC 10049	+	+	−	−
Clostridium difficile	NCTC 11209	+	+	−	−
Acholeplasm laidlawii	NCTC 10116	+	+	−	−
Mycoplasma fermentans	NCTC 10117	+	+	−	−
Mycoplasma hyorhinis	NCTC 10130	+	+	−	−
Mycoplasma orale	NCTC 10112	+	+	−	−
Mycoplasma hominis	NCTC 10111	+	+	−	−

TABLE 3. Nucleotide sequences of primers and probes

Primer or probe code	Sequence	Location[a]	Fluorophore/quencher
HJ-plasmid-1	5′-AACCAAGGTCGATGTGATAG-3′	7179	
HJ-plasmid-2	5′-TCAGATAATTGGCGATTCTT-3′	7328	
HJ-plasmid probe	5′-CGAACTCATCGGCGATAAGG-3′	7219	ROX/BHQ2
HJ-MOMP-1	5′-GACTTTGTTTTCGACCGTGTT-3′	199	
HJ-MOMP-2	5′-ACARAATACATCAAARCGATCCCA-3′	417	
MGB-MOMP probe[b]	5′-ATGTTTACVAAYGCYGCTT-3′	368	VIC/NFQ
Internal control forward primer	5′-GTGCTCACAC CAGTTGCCGC-3′		
Internal control reverse primer	5′-GCTTGGCAGC TCGCATCTCG-3′		
Internal control probe	5′-ATTGTGTGG GTGTGGTGTGGGTGTGTGC-3′		CY5/BHQ3
Internal control synthetic DNA	5′-GTGCTCACAC CAGTTGCCGC GGAAAGTATG TGGAATGTTA ACACACCCAC ACCACACCCA CACACGTGTT GGATCAATTT CGAGATGCGA GCTGCCAAGC-3′		
Primers for limiting dilution nested PCR for MOMP gene			
Outer forward primer	5′-TTGTTTTCGACCGTGTTTTG-3′	203	
Outer reverse primer	5′-AGCRTATTGGAAAGAAGCBCCTAA-3′	657	
Inner forward primer	5′-AAACWGATGTGAATAAAGARTT-3′	223	
Inner reverse primer	5′-TCCCASARAGCTGCDCGAGC-3′	617	
Primers for limiting dilution nested PCR for cryptic plasmid			
Outer forward primer	5′-TTGGCYGCTAGAAAAGGCGATT-3′	7153	
Outer reverse primer	5′-TCCGGAACAYATGATGCGAAGT-3′	7365	
HJ-plasmid-1	Sequence shown above		
HJ-plasmid-2	Sequence shown above		

[a] The positions of the primers and the probes for MOMP and cryptic plasmid components of the assay are according to GenBank accession nos. AF202457 and X06707, respectively.
[b] Additional labeling of the probe with the MGB molecule at the 3′ and to increase the melting temperature of the DNA duplex.

Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies, Paisley, United Kingdom), 6.25 pmol of each primer, and 2.5 pmol of each probe for amplification of DNA from the CP and the MOMP gene of *C. trachomatis* and six molecules of the IC and 2.5 pmol of each primer and probe for amplification of the IC. The IC was an artificial single-stranded DNA molecule that was designed by joining A, C, G, and T in a random order. The nucleotide-nucleotide BLAST program at www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi demonstrated the unique sequence of this molecule. Metabion quantified it by spectrophotometery, and the number of molecules/μl was calculated by using Avogadro's number. The amplification reaction profile included heating at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 1 s and 60°C for 60 s. The acquisition of a signal was preformed at 60°C during each cycle.

Quantification standards. Cryptic plasmid and MOMP gene copy numbers in a stock of purified DNA from *C. trachomatis* strain E (BOUR) were estimated by the limited-dilution, Poisson distribution method by a nested PCR (22). The nested PCRs for the CP and MOMP genes were performed as described previously (12). The sequences of the primers are shown in Table 3. An external calibration curve was prepared by using serial 10-fold dilutions from the stock DNA in nuclease-free water containing 1 ng/μl poly(A) RNA (Amersham Bioscience United Kingdom Ltd., Chalfont St. Giles, United Kingdom). The Rotor-Gene software calculated the number of *C. trachomatis* genome copies in amplification reactions with reference to the external calibration curve. The parameters used for quantification analysis are shown in Table 4.

TABLE 4. Parameters of Rotor-Gene 3000 for quantification analysis

Parameter	Value
Threshold	0.03
Left threshold	10
Start normalizing	Variable (generally from cycle 5)
Reaction efficiency threshold	Disabled
Normalization method	Dynamic tube normalization
No-template control threshold	10%

Criteria for a positive result by real-time PCR. A sample was considered positive for *C. trachomatis* DNA if it produced signals in the channels for the CP and the MOMP components of the assay. If the signal was produced in only one of these channels, the test was repeated, in duplicate, with the DNA extract. The sample was designated positive if repeat testing confirmed the earlier finding. Otherwise, it was designated a false-positive result. The algorithm for interpretation of the assay results is shown in Fig. 1.

Design of experiments. The lower limits of detection for the MOMP and the CP components of the assay were determined by testing a doubling dilution series of the positive control with from 25 to 0.19 genome copies per reaction mixture. The CP DNA copy number was four times greater than the MOMP gene copy number in each reaction mixture. Each concentration was tested in 40 replicates, and probit analysis of the results was carried out by using "StatsDirect," version 2.4.1 (StatsDirect, Ltd., Cheshire, United Kingdom). The analytical specificity of the assay was investigated by performing PCR with DNA from the chlamydial and bacterial strains stated in Tables 1 and 2. Intra- and interassay reproducibilities were investigated in three different runs by using DNA from the positive control. Ten replicates of five serial 10-fold dilutions ranging from 25 to 250,000 genome copies per reaction mixture were included in each run. Intra- and interassay reproducibilities were also investigated for 14 clinical samples. A total of six replicates of each sample were included in two different runs. The results from the intra- and interassay reproducibility experiments were used to investigate the linearity and the dynamic range of the MOMP component of the assay.

RESULTS

Analytical sensitivity and specificity. According to probit analysis, the assay demonstrated a 95% probability of detection with three MOMP gene copies and one CP copy per reaction mixture. The assay was able to detect DNA from all strains of *C. trachomatis* listed in Table 1. No amplicon was generated in the *C. trachomatis*-specific components of the assay from DNA of *C. suis*, *C. pneumoniae*, or the bacterial

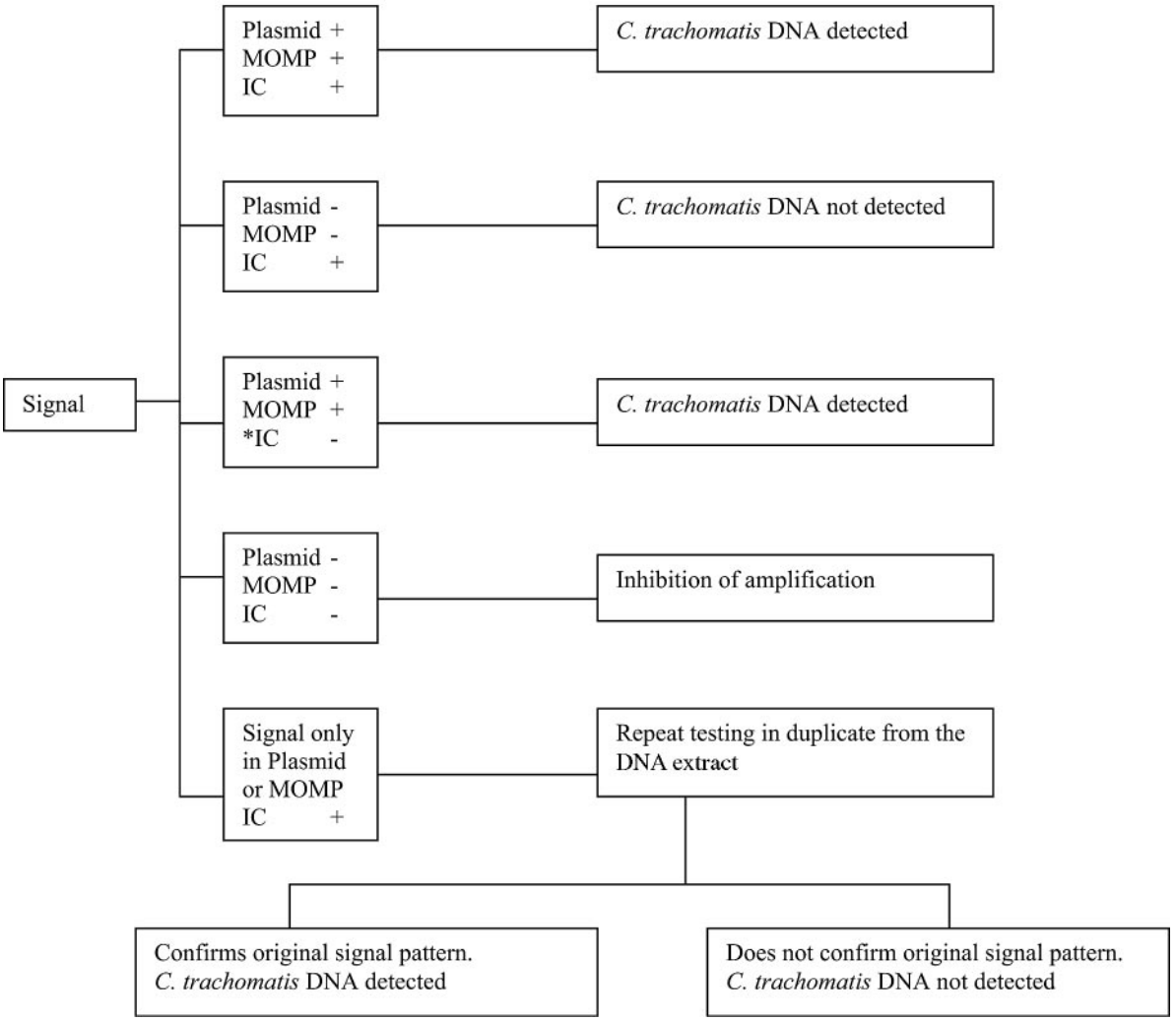

FIG. 1. Algorithm for interpretation of assay results. Plasmid, MOMP, and IC represent the three components of the assay. *, high chlamydia genome copy number in the reaction may suppress amplification of the IC.

isolates (Table 2). The 16S rRNA PCR amplified DNA from all bacteria shown in Table 2.

Intra- and interassay reproducibilities and dynamic range. The results of the intra- and interassay reproducibilities for the positive controls are shown in Fig. 2. The coefficient of variation (CV) values for across run, within run, and total variation generally increased with decreasing genome copy numbers in the reaction mixture for both the positive control and the clinical samples. The CV values for the positive control and the clinical samples are shown in Table 5 and Table 6, respectively. The data from the intra- and interassay reproducibility experiments were used to investigate the linearity of the assay. The MOMP component of the assay exhibited a log-linear response, characteristic of levels of *C. trachomatis* genome input between 25 and 250,000 copies per reaction mixture. The regression line of the observed concentration to the expected concentration on a $\log_{10}$ scale is shown in Fig. 3. For this set of data, the adjusted r^2 value was 0.99 and the deviation from linearity was 0.3 $\log_{10}$.

Validation of the assay. According to the criteria stated in the Materials and Methods section, the assay detected *C. trachomatis* DNA from all of the 146 Amplicor PCR-positive samples. It did not detect *C. trachomatis* DNA from any of the 122 Amplicor PCR-negative samples. Of 146 (96%) samples, 140 generated signals in both *C. trachomatis*-specific components of the assay. Six samples generated a signal only in the CP component of the assay. Repeat testing confirmed the presence of CP DNA and absence of *C. trachomatis* genomic DNA in these samples. The assay was able to quantify the *C. trachomatis* load in 95 (69%) of 140 samples. The median *C. trachomatis* load in these samples was 321 genome copies per reaction mixture (range, 26 to 40,137 genome copies per reaction mixture). The IC was detected in all of the 268 samples and the controls. A weak signal was observed after >35 cycles of amplification in the MOMP component of the assay for one Amplicor PCR-negative sample. A similar signal was also observed in the CP component of the assay for two of the Amplicor PCR-negative samples. The signals in these three sam-

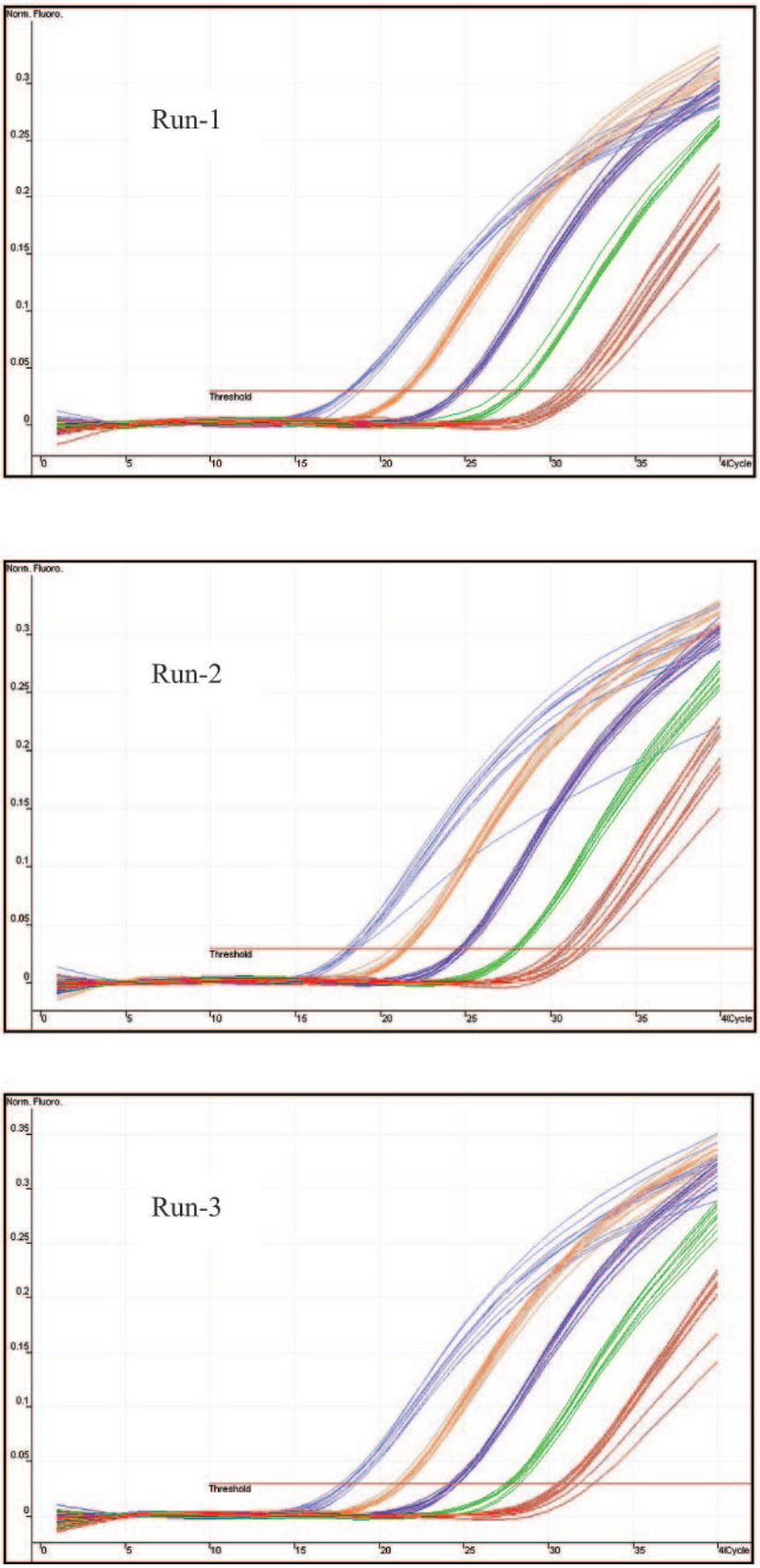

FIG. 2. Intra- and interassay reproducibilities. Ten replicates of each of 10-fold serial dilutions of *C. trachomatis* DNA were included per run. Color code: blue, 250,000 genome copies per reaction mixture; orange, 25,000 genome copies per reaction mixture; purple, 2,500 genome copies per reaction mixture; green, 250 genome copies per reaction mixture; red, 25 genome copies per reaction mixture.

TABLE 5. Intra- and interassay reproducibilities of the assay with DNA from *C. trachomatis* strain E (BOUR)[a]

Concn (no. of genome copies/reaction)	% CV		
	Run to run	Within run	Total
250,000	19	2	19
25,000	16	5	17
2,500	13	6	14
250	18	1	18
25	38	4	39

[a] Thirty replicates were performed.

ples were not detected on repeat testing and were considered nonspecific reactivity due to autohydrolysis of the probe. These samples were designated negative for *C. trachomatis* DNA after repeat testing.

DISCUSSION

The two previously reported in-house real-time PCR assays for the detection of *C. trachomatis* were qualitative in nature (8, 13). This is the first assay that can be used for both the qualitative and the quantitative detection of *C. trachomatis* in clinical samples. At present, quantification of *C. trachomatis* is not considered valuable for the clinical management of an infection. However, it may be helpful in providing an understanding of the pathogenesis and the dynamics of the infection, i.e., the degree of infectivity, the severity of disease, the risk of developing sequelae, and the response to therapy.

There are multiple copies of CP in *C. trachomatis*. The majority of NAATs use it as the target for amplification to enhance the sensitivity of detection. CP is detected among all genotypes of *C. trachomatis*, and its nucleotide sequence is highly conserved (16). However, a number of studies have described clinical (9, 18, 20, 24) and laboratory (14) isolates of *C. trachomatis* that lack CP, suggesting that it is not essential for growth of the organism. In assays targeting CP alone, there is a risk of producing false-negative results (16), the magnitude of which is not known at present. Furthermore, CP is an inappropriate target for the quantification of *C. trachomatis* in clinical samples because of its variable number in the organism. Assays targeting chromosomal genes of *C. trachomatis* can overcome these two problems, albeit with a slightly reduced sensitivity. Six (4%) of 146 positive samples were detected by identifying the CP component of the assay only, proving its value as a target in NAATs. Thus, CP was chosen as a target for the assay described here. The MOMP gene was used to rule out the possibility of a false-negative result due to the absence of CP in *C. trachomatis* and to quantify the infection.

TABLE 6. Intra- and interassay reproducibilities of the assay for 14 clinical samples[a]

Sample no.	Mean concn (no. of genome copies/reaction)	% CV		
		Run to run	Within run	Total
1	37,496	18	10	21
2	28,939	15	9	18
3	5,808	18	6	19
4	5,263	23	4	23
5	1300	13	11	18
6	1016	12	3	12
7	75	26	6	26
8	72	42	4	42
9	21	35	65	74
10	17	51	6	52
11	13	57	46	73
12	11	54	11	55
13	5	103	43	111
14	5	48	1	48

[a] Six replicates were performed.

A number of studies have reported reproducibility problems with commercial NAATs for the diagnosis of *C. trachomatis* infection (3, 5, 6, 7, 13, 17), and a repeat of the same assay with the initial extract or DNA newly extracted from the same sample did not improve the accuracy of diagnosis (5, 6, 13). Since our new assay targeted two different genes of chlamydia, the presence or absence of an amplification signal in one chlamydia-specific component of the assay was confirmed by the presence or the absence of a signal in the corresponding chlamydia-specific component of the assay. Of 268 results, 259 (97%) were confirmed without repeat testing. Repeat testing was performed with nine samples due to the generation of a very weak signal in only one chlamydia-specific component of the assay. There could be two possible explanations for this phenomenon: a very low quantity of target (true weak-positive result) or autohydrolysis of the probe (false-positive result). Transposition errors and cross contamination were unlikely due to the automated extraction of the DNA in a closed system. Repeat testing of the already extracted DNA identified six of nine samples as having true-positive results and three samples as having false-positive results. Since the Amplicor assay, the reference test, confirmed these results, repeat testing was not done with the original samples. However, reextraction of DNA from the original samples may be needed if repeat testing with DNA that was already extracted is unable to resolve the true nature of a weak signal in one of the chlamydia-specific components of the assay. All samples in the validation panel were correctly identified by the assay, which demonstrated a 100% correlation with the Amplicor PCR results. The data presented in this study have demonstrated the excellent analytical performance of the assay. However, the number of samples in the validation panel was relatively small, and validation of the assay was performed in research settings. Further work is needed to investigate the clinical performance of the assay with a much larger number of samples in a routine clinical diagnostic laboratory.

The assay detected all genovars of *C. trachomatis*, including genovars for lymphogranuloma venereum and trachoma. No cross-reactivity of the primers and the probes was observed for the pathogenic bacteria or the commensal organisms of the oropharynx and the genital tract. The assay demonstrated a wide dynamic range for quantification. The within-run CV was less than 6% for all dilutions with from 25 to 250,000 genome copies per reaction mixture. While the run-to-run CV was less than 19% for dilutions with from 250 to 250,000 genome copies per reaction mixture, this value was 38% for 25 genome copies per reaction mixture. The high CV for a low copy number is a common feature of all quantitative assays. Simultaneous amplification of three targets in a single reaction may have some effect on the reproducibility of the assay for quantification. No

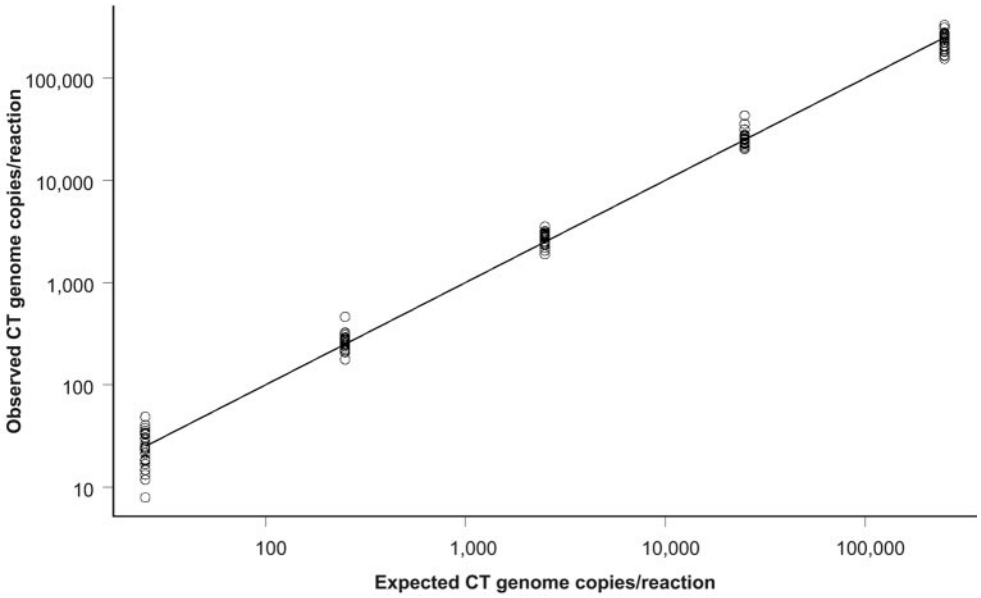

FIG. 3. Linearity of the assay. Data are from three 10-fold dilution panels of *C. trachomatis* (CT) DNA (10 replicates each) ranging from 25 to 250,000 genome copies per reaction mixture.

such effect was observed for qualitative detection of *C. trachomatis* DNA.

Due to the detection and confirmation of *C. trachomatis* DNA in a single reaction, this assay is ideally suited for laboratories with high workloads. DNA extraction and amplification are already automated in this assay. We are currently testing different platforms to automate this assay fully. Investigations are under way to evaluate its performance in comparison with that of Amplicor PCR for 1,000 consecutive patients attending a genitourinary medicine clinic. Further studies are planned to investigate the effectiveness of this assay in detecting *C. trachomatis* DNA in urine and rectal, pharyngeal, and eye swab specimens. Only three of the four channels available in the Rotor-Gene were used for this assay. The fourth channel could potentially be used for the detection of *Neisseria gonorrhoeae*, along with *C. trachomatis*, in the same reaction at a negligible cost. Although quality control for the DNA extraction step was performed by the inclusion of positive and negative controls, the efficiency of extraction was not monitored for individual samples. We plan to genetically engineer an *Escherichia coli* strain containing the unique internal control used in this assay. This strain will be used to monitor the efficiency of all steps in the assay for every individual sample.

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